# Regulation of *Limulus* skeletal muscle contraction

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**Abstract** **Skeletal muscle contraction of *Limulus polyphemus*, the horseshoe crab, seemed to be regulated in a dual manner, namely $Ca^{2+}$ binding to the troponin complex as well phosphorylation of the myosin light chains (MLC) by a $Ca^{2+}$/calmodulin-dependent myosin light chain kinase. We investigated muscle contraction in *Limulus* skinned fibers in the presence of $Ca^{2+}$ and of $Ca^{2+}$/calmodulin to find out which of the two mechanisms prevails in *Limulus* skeletal muscle contraction. Although skinned fibers revealed high basal MLC mono- and biphosphorylation levels (0.48 mol phosphate/mol 31 kDa MLC; 0.52 mol phosphate/mol 21 kDa MLC), the muscle fibers were fully relaxed at pCa 8. Upon $C^{2+}$ or $Ca^{2+}$/calmodulin activation, the fibers developed force ($357 \pm 78.7$ mN/mm$^2$; $338 \pm 69.7$ mN/mm$^2$, respectively) while the MLC phosphorylation remained essentially unchanged. We conclude that $Ca^{2+}$ activation is the dominant regulatory mechanism in *Limulus* skeletal muscle contraction.**



*Key words:* Myosin light chain; Skeletal muscle; $Ca^{2+}$; Calmodulin; *Limulus*

## 1. Introduction

In cardiac, skeletal and smooth muscle the myosin light chain 2 (MLC-2; regulatory MLC) can be phosphorylated reversibly by a $Ca^{2+}$/calmodulin-dependent myosin light chain kinase (MLCK) and a myosin light chain phosphatase (MLCP) [1]. In scallop muscle and in smooth muscle phosphorylation of MLC-2 initiates contraction while in striated muscle MLC-2 phosphorylation increases calcium sensitivity of isometric force [2,3]. Skeletal muscle contraction in the horseshoe crab is supposed to be regulated by a dual system, namely $Ca^{2+}$ binding to troponin subunits as well as a calmodulin-dependent phosphorylation of the regulatory myosin light chains with an endogenous MLCK [4]. *Limulus polyphemus*, the horseshoe crab, expressed two phosphorylatable light chain isoforms with molecular weights of 31 kDa and 21 kDa. A 19 kDa MLC is not phosphorylated [5]. Phosphorylation of *Limulus* myosin initiated actin filament sliding in the in vitro motility assay [6]. However, it was also shown that a change in phosphorylation is not responsible for $Ca^{2+}$-activated tension [7]. Despite these data it could not be finally revealed which of the two regulatory mechanisms in *Limulus* is dominant.

In this work, we investigated the impact of phosphorylation of MLC-2 on skeletal muscle contraction in *Limulus*. Phosphorylation of the regulatory MLCs was determined during relaxation and during maximum $Ca^{2+}$-activated isometric contraction. If phosphorylation is of major importance for initiation of contraction, we expected to change the extent of the phosphorylation of the MLC due to $Ca^{2+}$ activation.

## 2. Materials and methods

### 2.1. Tissue preparation

Skeletal muscle was prepared from *Limulus* telson levator muscle. Briefly, muscle fiber bundles with a diameter of approx. 0.3 mm and a length of 5–10 mm were dissected from the base of the tail region. The insertion and the end of the fiber were fixed. The muscle was held isometric in relaxation solution and stretched slightly. For the preparation of the skinned fibers, the muscle fiber bundles were further dissected into 5–10 mm long fibers which were approximately 150 μm in diameter. These fibers were then incubated in a solution containing 20 mmol/l imidazole, 10 mmol/l $NaN_3$, 5 mmol/l ATP, 5 mmol/l $MgCl_2$, 4 mmol/l EGTA, 2 mmol/l DTE, 50% glycerol, 1% Triton X-100, pH 7, at 4°C for 20 h. Subsequently the fibers were transferred into the same solution except Triton X-100 and stored at −20°C.

### 2.2. Biochemical analysis

All biochemical experiments were performed with demembranated multicellular skeletal muscle fibers (skinned fibers) from *Limulus* telson levator muscle prepared as described previously [8]. Myosin light chains were analyzed by a high-resolution two-dimensional gel electrophoresis (2D PAGE) technique. Isoelectric focusing (first dimension) was performed in glass capillaries (12.5 cm long, 1 mm inner diameter) using the pH gradient 4.5–5.5 (Pharmalytes; Pharmacia, Sweden). The gels were run overnight at 600 V constant load for the first dimensional separation. The second dimension was SDS electrophoresis, using slab gels of $10.5 \times 9.5$ cm, 1 mm thick. The gels were stained in Coomassie blue and the MLC were evaluated by computer-assisted scanner densitometry (ScanPack, Biometra, Germany). Densitometric analysis of the MLC was always within the linear portion of the relation between protein concentration and optical density (Lambert-Beer law).

### 2.3. Autoradiography

Phosphorylation of the different isoforms of the regulatory MLC in *Limulus* telson levator muscle was investigated by radiography. Skeletal muscle fiber bundles were demembranated in 0.5% Triton X-100. Subsequently the 'skinned' muscle fiber bundles were incubated in contraction solution (pCa 4.5), containing radioactive [$\gamma$-$^{32}$P]ATP, 250 μCi. After 30 min at room temperature incorporation of $^{32}$P was stopped by trichloroacetic acid (TCA). The incubation was performed at room temperature. The three different isoforms of the MLC of *Limulus* were then separated by 2D PAGE. A X-ray film (Osray M3, Agfa-Gevaert, Belgium) was exposed to the radioactive gel for 5 days.

### 2.4. Mechanical analysis

For all mechanical experiments, skeletal muscle fibers were dissected into bundles of 150 μm in diameter and 7 mm in length under a preparation microscope. Fibers were mounted isometrically between a force transducer and a length step generator with microsyringes in relaxation solution. Relaxation solution (pCa 8) contained: imidazole 25 mM, ATP 10 mM, creatine phosphate 10 mM, $MgCl_2$ 12.5 mM, $NaN_3$ 5 mM, DTE 1 mM, EGTA 5 mM, KCl 12.5 mM, creatine kinase 380 U/ml, pH 7. Contraction solution (pCa 4.5) was the same as relaxation solution except that EGTA was replaced with 5 mM CaEGTA. Fibers for biochemical analysis were taken after contraction and relaxation from the respective solutions. In the solutions containing calmodulin the concentration was $10^{-6}$ M. For

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exogenous calmodulin substitution we used a stock solution with 1.68 mg calmodulin per 100 ml distilled water. In each case, fibers were fixed in TCA, 4°C, to stop phosphorylation.

## 3. Results

### 3.1. Myosin light chain analysis

The MLC isoforms were analyzed according to their isoelectric point and molecular weight by 2D PAGE. We detected three isoforms of the myosin light chains in the horseshoe crab. The 31 kDa and the 21 kDa MLC could be separated into three different protein spots, having identical molecular weights but different isoelectric points (Fig. 1). A 19 kDa MLC existed as a single protein (Fig. 1).

### 3.2. Light chain phosphorylation

For detection of the phosphorylatable MLC isoforms, *Limulus* skeletal muscle fibers were incubated in contraction solution containing [$\gamma$-$^{32}$P]ATP, 250 μCi for 20 min. MLCs were separated by 2D PAGE and visualized by autoradiography. $^{32}$P was incorporated into the two most acidic 31 kDa and 21 kDa isoforms but not into the 19 kDa isoform (Fig. 2).

### 3.3. $Ca^{2+}$ and calmodulin dependence of Limulus MLC phosphorylation

$Ca^{2+}$ dependence of the phosphorylation of the regulatory MLC in *Limulus* was under investigation. To evaluate the extent of the phosphorylation of the 31 and the 21 kDa MLCs, phosphorylation was stopped by TCA after 1, 5 and 15 min in relaxation solution (pCa 8) and, in another set of experiments, in contraction solution (pCa 4.5) during isometric steady-state tension. However, time had no influence on phosphorylation (data not shown). The relation of the different isoforms and the extent of the phosphorylation was determined by Coomassie blue-stained 2D polyacrylamide gels. The values are means ± S.E.M. of six different fibers. Following $Ca^{2+}$ activation (pCa 4.5), there was no significant change of phosphorylation of the mono- and biphosphorylated forms of the 31 and 21 kDa MLC isoforms (Fig. 3). During relaxation the ratio of the un-, mono- and biphosphorylated isoforms of the 31 kDa MLC were 52 ± 6%, 26 ± 6%, 22 ± 5% (100% = all three isoforms of the 31 kDa MLC), during contraction it was 50 ± 6%, 28 ± 7%, 22 ± 4%. The ratios of the three isoforms of the 21 kDa MLC were 48 ± 3%, 39 ± 6%, 13 ± 3% during relaxation and 53 ± 4%, 36 ± 3%, 11 ± 6% during $Ca^{2+}$-activated contraction.

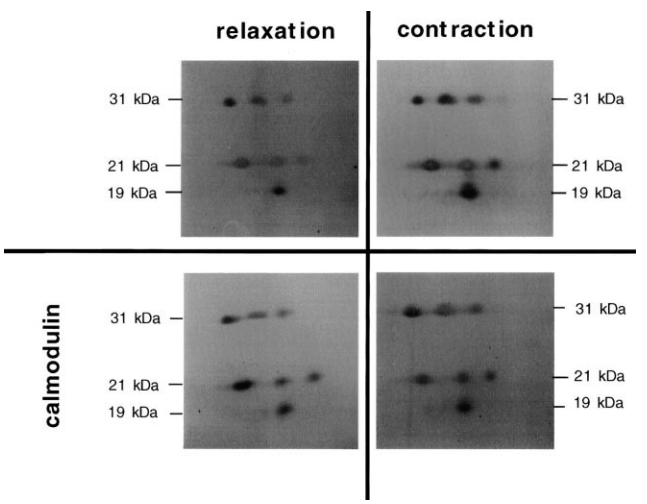


Fig. 1. MLC separation by 2D PAGE. Original photographs of 2D electrophoresis gels of *Limulus* skeletal muscle fibers. The 31 kDa and the 21 kDa isoforms of the myosin light chains could be separated into three single protein spots along with rising acidity. The 19 kDa isoform could not be further separated. The separation was the same during relaxation (gels on the left) or contraction (gels on the right) and with (bottom) and without (top) calmodulin.

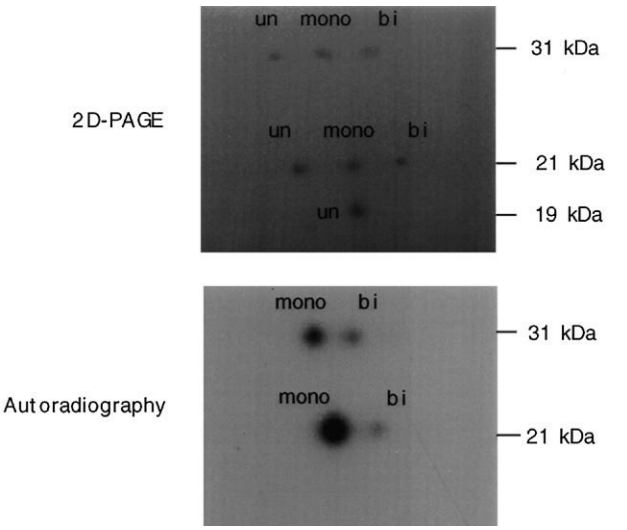


Fig. 2. Autoradiography (bottom) of a 2D electrophoresis gel (top) revealed that the two most acidic spots of the 31 kDa and the 21 kDa MLC isoforms could be mono- and biphosphorylated, respectively, whereas the 19 kDa isoform is unphosphorylated. For evaluation of MLC phosphorylation fibers were incubated with [$\gamma$-$^{32}$P]ATP, subsequently MLCs were separated by 2D electrophoresis and a X-ray film was exposed to the radioactive gel. Un, unphosphorylated; mono, monophosphorylated; bi, biphosphorylated.

$Ca^{2+}$/calmodulin dependence of the phosphorylation was investigated in a second approach. Phosphorylation of the regulatory light chains was stopped by TCA during relaxation or contraction. Relaxation (pCa 8) and contraction (pCa 4.5)

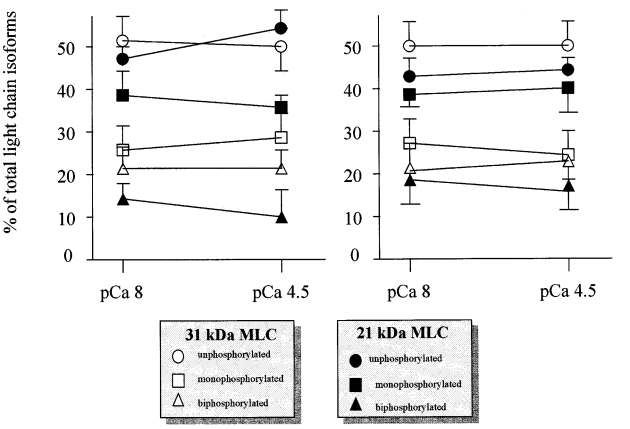


Fig. 3. Comparison of MLC phosphorylation. Extent of phosphorylation of the regulatory MLC (21 and 31 kDa isoforms). The isoforms are either un-, mono- or biphosphorylated. Phosphorylation was determined during relaxation or contraction. Amounts of single MLC isoforms were evaluated by computer-assisted scanner densitometry. Left side: values without calmodulin. Right side: values with calmodulin. The values for the un-, mono- or biphosphorylated isoforms are given in % of the total amount of the respective isoform (100% = un+mono+biphosphorylated spots of each single isoform). There was no statistically significant difference between the respective isoforms (*t*-test; $n = 6$ for each value).

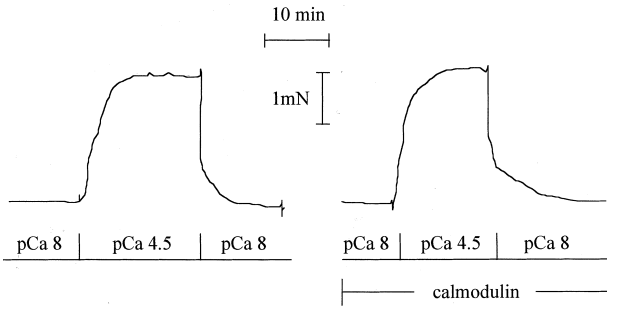


Fig. 4. $Ca^{2+}$/calmodulin dependence of force generation. Drawn after original registration of force generation upon $Ca^{2+}$- (left) or $Ca^{2+}$/calmodulin- (right) activated contraction of *Limulus* skeletal muscle fibers (telson levator). Force production without calmodulin ($357 \pm 78.7$ mN/mm$^2$; $n = 6$) was statistically not significantly different from force production with calmodulin ($338 \pm 69.7$ mN/mm$^2$; $n = 6$).

solutions were the same as before with additional calmodulin ($10^{-6}$ M). The activation of the *Limulus* skeletal muscle fibers by $Ca^{2+}$ and calmodulin revealed no effect on the phosphorylation of the regulatory MLC in *Limulus*. The phosphorylation of the regulatory MLC was not significantly different during relaxation or contraction (Fig. 3). During relaxation the ratio of the un-, mono- and biphosphorylated isoforms of the 31 kDa MLC were $50 \pm 5\%$, $28 \pm 6\%$, $22 \pm 7\%$ (100% = all three isoforms of the 31 kDa MLC), during contraction it was $50 \pm 6\%$, $26 \pm 5\%$, $24 \pm 4\%$. The ratios of the three isoforms of the 21 kDa MLC were $43 \pm 3\%$, $39 \pm 2\%$, $18 \pm 6\%$ during relaxation and $44 \pm 2\%$, $40 \pm 7\%$, $16 \pm 5\%$ during $Ca^{2+}$/calmodulin-activated contraction, respectively. There was no difference in the mechanical analysis of skinned *Limulus* skeletal muscle fibers. Tension development upon $Ca^{2+}$ activation was about the same without ($357 \pm 78.7$ mN/mm$^2$) and with ($338 \pm 69.7$ mN/mm$^2$) calmodulin (Fig. 4).

## 4. Discussion

In vertebrate skeletal muscle an increase in phosphorylation of the regulatory myosin light chains increased the rate of the switch of the crossbridges from the non-force-generating to the force-generating state ($f_{app}$) as well as $Ca^{2+}$ sensitivity of isometric tension development [9]. There was no influence on the detachment rate ($g_{app}$) [9]. Contraction of the *Limulus* skeletal muscle is believed to be regulated in a dual manner [4]: besides a $Ca^{2+}$-activated troponin-tropomyosin mechanism, a calmodulin-dependent phosphorylation of the regulatory MLC is supposed to initiate contraction [5,10]. In contrast, other authors suggested that a calmodulin-dependent phosphorylation of the regulatory MLC in *Limulus* causes only a modulation of the force generation [7]. Due to these divergent points of view, we investigated simultaneously force and phosphorylation of the regulatory myosin light chains in *Limulus* upon $Ca^{2+}$ activation with and without calmodulin. In accordance with previous studies [5,6] we found two phosphorylatable MLCs with molecular weights of 31 kDa and 21 kDa. For the first time we could demonstrate in this work that both isoforms are mono- and biphosphorylated. Our skeletal muscle fibers were demembranated to avoid diffusion problems and to remove endogenous MLCK. We did not substitute MLCK since we did not want to change phosphorylation levels during contraction. Indeed, phosphorylation of the two phosphorylatable isoforms did not change during relaxation or $Ca^{2+}$- and $Ca^{2+}$/calmodulin-activated contraction, suggesting the presence of MLCK in the soluble cytoplasmic compartment similar to mammalian striated muscle [1]. MLC isoforms were already phosphorylated during relaxation. Since we did not add exogenous MLCK to the demembranated muscle fibers, the extent of phosphorylation did not change during contraction. Thus, we demonstrated that contraction could be performed without alterations in MLC isoform phosphorylation. Since initiation of force development could be elicited by $Ca^{2+}$ without changes of phosphorylation of the 31 and the 21 kDa MLCs, tension development in *Limulus* skeletal muscle seems to be initiated by $Ca^{2+}$ binding to troponin C rather than by phosphorylation of MLC-2 isoforms. Our data demonstrate that phosphorylation of the regulatory MLC is not a prerequisite for the regulation of force generation. Rather, in analogy to mammalian striated muscle [3,10], we suggest a modulatory role of $Ca^{2+}$ sensitivity by MLC phosphorylation in *Limulus* skeletal muscle.

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